14. The TGF-α/EGF radioreceptor assay was performed with a kit from Biomedical Technology, Inc., in accordance with the manufacturer's instructions.
15. Z. Werb and J. R. Chin, *J. Biol. Chem.* **258**, 10642 (1983); Z. Werb and J. R. Chin, *J. Exp. Med.* **158**, 1272 (1983).
16. K. Siminoski, J. Bernanke, C. Kay, R. A. Murphy, *Endocrinology* **118**, 1417 (1986); D. Clegg, L. Reichardt, D. A. Rappolee, Z. Werb, manuscript in preparation.
17. Oligonucleotide sequences are designed from the following cDNA sequences: β-actin, S. Alonso, A. Minty, Y. Bourlet, M. Buckingham, *J. Mol. Evol.* **23**, 11 (1986); TGF-β, D. C. Lee, T. M. Rose, N. R. Webb, G. J. Todaro, *Nature* **313**, 489 (1985); TGF-α, R. Derynck, J. A. Jarrett, E. Y. Chen, D. V. Goeddel, *J. Biol. Chem.* **261**, 4377 (1986); PDGF-A, C. Betsholtz *et al.*, *Nature* **320**, 695 (1986); EGF, A. Gray, T. J. Dull, A. Ullrich, *ibid.* **303**, 722 (1983); IL-1α, P. T. LoMedico *et al.*, *ibid.* **312**, 458 (1984); IGF-1, G. I. Bell *et al.*, *Nucleic Acids Res.* **20**, 7873 (1986). Whenever possible, the positions of the oligonucleotide primers are chosen to include a diagnostic restriction endonuclease site and an intron so that PCR fragments generated from any contaminating genomic DNA can be distinguished from those generated from the mRNA (*11*).
18. J. L. Goldstein, Y. K. Ho, S. K. Basu, M. S. Brown, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 333 (1979); M. S. Brown and J. L. Goldstein, *Nature* **316**, 680 (1985); R. Takemura and Z. Werb, *J. Exp. Med.* **159**, 167 (1984); R. Ross, *N. Engl. J. Med.* **314**, 488 (1986).
19. Mitogenesis was assayed by determining [^{3}H]thymidine uptake into quiescent confluent Balb/c 3T3 fibroblasts. Fetal calf serum (10%) was used as a positive control, and 10% RPMI 1640 was used as a negative control for the mitogenesis assay by the methods of W. Wharton, G. Y. Gillespie, S. W. Russell, W. J. Pledger, *J. Cell. Physiol.* **110**, 93 (1982).
20. Y. Shing *et al.*, *Science* **223**, 1296 (1984).
21. T. A. Mustoe *et al.*, *ibid.* **237**, 1333 (1987).
22. S. M. Wahl *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5788 (1987).
23. For the dot immunoblot assay [P. V. Talbot, R. L. Knobler, M. J. Buchmeier, *J. Immunol. Methods* **73**, 177 (1984)] basic FGF column fraction samples or 1 to 10,000 pg of basic FGF or acidic FGF (a gift of D. Gospodarowicz) were dotted onto nitrocellulose, dried, and blocked with 3% normal rabbit serum, then with 1/1000 dilution of a monoclonal antibody to basic FGF (a gift of D. Gospodarowicz), developed with biotinylated rabbit antibody to mouse IgG (Hyclone) diluted 1/1000, followed by alkaline phosphatase-labeled avidin (Vector) at 0.2 U/ml. For immunodot assays of PDGF, samples of macrophage-conditioned medium or 0.01 to 1.0 U of PDGF (Collaborative Research) were dotted onto nitrocellulose, dried, blocked with 3% normal goat serum, then incubated with 1/500 dilution of goat polyclonal antibody to PDGF (a gift of G. Grotendorst) and developed with biotinylated sheep antibody to goat IgG (Sigma) at 1/1,000, followed by alkaline phosphatase–labeled avidin (Vector), nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate (Sigma). The TGF-β immunodot assay was performed in a similar manner with anti-TGF-β (R&D Systems, Inc.).
24. D. Mark, unpublished results.
25. For immunofluorescence staining, adherent cells from wound cylinders or TEPM on 12-mm glass cover slips were fixed for 10 to 30 min in freshly prepared 2% paraformaldehyde, then washed in PBS containing 0.1% glycine. Cells to be stained with F4/80, a macrophage-specific rat monoclonal antibody [J. M. Austyn and S. Gordon, *Eur. J. Immunol.* **11**, 805 (1981)] were left unpermeabilized. Cells to be stained with anti–TGF-α (Peninsula Laboratories) were permeabilized with 0.25% Triton X-100 for 4 min. Nonspecific binding sites were then blocked with ovalbumin (1 mg/ml) for 30 min, followed by 5% skim milk powder for 30 min. Samples were then stained for 1 hour with F4/80 or nonimmune rat IgG at 20 μg/ml, or with anti–TGF-α or nonimmune rabbit serum at 1:100, diluted in 5% skim milk. After extensive washing, cells were then incubated with biotinylated F(ab′)$_2$ fragments from either anti-rat IgG or anti-rabbit IgG (Hy-Clone), diluted 1:100, washed, and stained with Texas red–labeled streptavidin (Amersham). Cells were observed with a 63× water-phase PlanNeofluor lens on a Zeiss Photomicroscope III and photographed on Tri-X film rated at 800 ASA for 60-second exposures.
26. P. Ralph, in *Mononuclear Phagocytes: Functional Aspects*, R. van Furth, Ed. (Nijhoff, The Hague, 1980), pp. 439–456; Z. Werb and J. R. Chin, *J. Cell Biol.* **97**, 1113 (1983).
27. RNA (20 μg) from LPS-stimulated or control P388D1 cells was fractionated on a 1% denaturing agarose gel, transferred to a nylon membrane, and cross-linked by ultraviolet irradiation for 3 min at 9 cm at 16 J/m^2 per second [G. M. Church and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1991 (1984)]. The blot was hybridized with a random-primed plasmid insert from mouse TGF-α cDNA (a gift of R. Derynck) and then rehybridized with a random-primed β-actin plasmid (a gift of M. Kirschner).
28. LPS-stimulated TEPM were radiolabeled with [^{35}S]cysteine (100 μCi/ml; specific activity, 1135 Ci/mmol) for 24 hours. Conditioned medium was processed as described (*13*) and was fractionated on a TSK 4000SW (30 cm) to 3000SW (60 cm) size exclusion column system equilibrated and run with a mobile phase of 0.1% trifluoroacetic acid–40% acetonitrile. The 3- to 20-kD fractions were immunoprecipitated with anti–TGF-α antiserum or nonimmune rabbit serum, separated by SDS–polyacrylamide gel electrophoresis on a 7 to 15% gradient gel under denaturing conditions, and then autoradiographed.
29. We thank K. S. Dwyer for implanting the wound cylinders, S. Gordon (University of Oxford) for the macrophage-specific rat monoclonal antibody and A. Wang, D. Gospodarowicz, G. Neufeld, P. Crocker, and D. Clegg for helpful comments. Supported by a contract from the Office of Health and Environmental Research, U.S. Department of Energy (DE-AC03-76-SF01012), NIH grants AR 32746 and GM 27345, and a University of California Regents Fellowship and University of California Patent Fund Award (to D.A.R.).

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Synthetic CD4 Peptide Derivatives That Inhibit HIV Infection and Cytopathicity

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Synthetic peptide segments of the CD4 molecule were tested for their ability to inhibit infection of CD4$^+$ cells by the human immunodeficiency virus (HIV) and to inhibit HIV-induced cell fusion. A peptide mixture composed of CD4(76–94), and synthesis side products, blocked HIV-induced cell fusion at a nominal concentration of 125 micromolar. Upon high-performance liquid chromatography, the antisyncytial activity of the peptide mixture was found not in the fraction containing the peptide CD4(76–94) itself, but in a side fraction containing derivatized peptide products generated in the automated synthesis. Derivatized deletion and substitution peptides in the region CD4(76–94) were used to demonstrate sequence specificity, a requirement for benzyl derivatization, and a core seven-residue fragment required for antisyncytial activity. A partially purified *S*-benzyl-CD4(83–94) peptide mixture inhibited HIV-induced cell fusion at a nominal concentration of ≤32 micromolar. Derivatized CD4 peptides blocked cell fusion induced by several HIV isolates and by the simian immunodeficiency virus, SIV, and blocked infection in vitro by four HIV-1 isolates with widely variant envelope gene sequences. Purified CD4(83–94) dibenzylated at cysteine 86 and glutamate 87 possessed antisyncytial activity at 125 micromolar. Derivatization may specifically alter the conformation of CD4 holoreceptor peptide fragments, increasing their antiviral efficacy.

LIGANDS THAT BIND TO, BUT DO NOT activate, biological receptors are called antagonists, and are used therapeutically as competitive inhibitors of ligand-receptor interactions (*1*). It should also be possible to use fragments of receptors as competitive inhibitors of ligand-receptor binding, provided that a small continuous region of a receptor binds the ligand and that this epitope, separated from the holoreceptor, has sufficient conformational stability to allow a thermodynamically favorable interaction with the ligand.

CD4 is a 55- to 58-kD glycoprotein originally characterized as a T lymphocyte differentiation antigen (*2*). It is implicated as an associative recognition element in major histocompatibility complex (MHC) class II–restricted immune responses (*3*) including the mixed leukocyte response (MLR) and antigen-specific MHC class II–restricted T

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cell cytotoxicity (*4*). CD4 also serves as a receptor for the primate immunodeficiency lentiviruses HIV-1, HIV-2, and SIV (*5*). Interactions between the envelope glycoprotein of HIV and CD4 are involved in HIV infection of susceptible cells including T lymphocytes and monocyte/macrophages; these interactions also mediate the characteristic cytopathic process of HIV-induced cell fusion (*6, 7*).

We have examined synthetic peptide fragments derived from the predicted amino acid sequence of the CD4 molecule (*8*) as potential inhibitors of CD4-dependent cellular functions. Peptide fragments of the extracellular domain of CD4 were synthesized (*9*) and tested for their ability to inhibit the MLR response (*3*), HIV-1– and SIV-induced cell fusion (*7, 10*) and HIV-1 and HIV-2 infection of $CD4^+$ cells (*11*). Peptides were initially tested as unpurified mixtures containing the desired peptide (65 to 95% of total product) as well as derivatized (incompletely deprotected) and sequence-terminated (deletion) peptides generated during automated synthesis. Peptides corresponding to CD4(1–25), CD4(26–50), CD4(51–75), and CD4(95–118), as well as CD4(261–285) and CD4(331–355), were completely inactive in inhibiting HIV-induced cell fusion (Table 1). The peptide mixture corresponding to CD4(76–94) was the only one in the series that inhibited HIV-induced cytopathology, completely blocking syncytium formation at a nominal concentration of 125 μ*M* (Table 1). CD4(76–94) peptide preparations also inhibited the infectivity of both HIV-1 and HIV-2 when tested in a quantitative syncytium-forming infectivity microassay (Table 1). The CD4(76–94) peptide mixture did not inhibit the MLR at the concentration that gave complete inhibition of HIV-induced cell fusion (Table 1).

Table 1. Biological effects of CD4 peptide fragments. The amino acid sequence shown (single-letter code) is that of the desired peptide. Material used was the post-resin peptide mixture (*9*). Several of the peptides were further purified by high-performance liquid chromatography, and the major peak was characterized by sequential Edman degradation or fast atom bombardment–mass spectrometry. None of the HPLC-purified peptides tested inhibited HIV-induced cell fusion at ≤500 μ*M*, the highest dose tested (see Fig. 1).

Peptide sequence	Fusion*	MLR†	Infection‡
QGNKVVLGKKGDTVELTCTASQKKS [CD4(1–25)]	NA	NA	NA
YQGNKVVLGKKGDTVELTCTASQKKS [YCD4(1–25)]	NA	NA	
IQFHWKNSNQIKILGNQGSTLTKGP [CD4(26–50)]	NA	NA	NA
YIQFHWKNSNQIKILGNQGSTLTKGP [YCD4(26–50)]	NA	NA	
YSKLNDRADSRRSLWDQGNFPLIIKN [CD4(51–75)]	NA	NA	NA
LKIEDSDTYICEVEDQKEE [CD4(76–94)]	125 μ*M*	NA	32 μ*M*
DQKEEELKIEDSDTYICEV [CD4(76–94), altered sequence]	NA		
VQLLVFGLTANSDTHLLQGQSLTL [CD4(95–119)]	NA		
LPQALPQYAGSGNLTLALELKTGKL [CD4(261–285)]	NA		
REKAVWVLNPEAGMWQCLLSDSGQV [CD4(331–355)]	NA		
ASTTTNYT [ARVgp120]	NA		NA
CEGNVRVSRELA [control peptide]	NA		
CKQLQKDKQVYR [control peptide]	NA		
CIDLGTDMVPAISLAY [control peptide]	NA		
GEWAHSRQEEEEMARAPQVLFRG [rat pancreastatin]	NA		

*For the fusion assay, 50 μl of peptide diluted in phosphate-buffered saline was added to 25 μl of medium containing 5×10^4 HIV-infected Hg cells ($Hg\text{-}HIV\text{-}1_{HXB2}$) and incubated at 37°C for 30 min; 25 μl of medium containing 5×10^4 $CD4^+$ indicator (VB) cells was then added, and cells were cocultured in the presence of peptide for 18 hours in a humid 5% CO_2 atmosphere. None of the peptides tested were cytotoxic as assessed by trypan blue exclusion. NA, not active. †The mixed leukocyte response (*3*) was measured in the presence or absence of peptide at about 125 μ*M*. None of the peptides caused a statistically significant decrease in T cell proliferation as measured by incorporation of tritiated thymidine, tested with three separate stimulator by responder cell populations. ‡The CEM-SS quantitative microtiter syncytia-forming assay was performed as described (*11*). The concentration given is that which results in a V_n/V_0 less than 0.005. [V_n/V_0 is the number of syncytia per well at confluence (5 to 6 days after viral infection) in the presence of peptide (V_n) divided by the number of syncytia per well at confluence in the absence of peptide (V_0)]. Viral inocula (100 to 200 syncytia-forming units of $HTLV\text{-}III_B$ (titered frozen stock from $HTLV\text{-}III_B$–infected H9 cells) were incubated for 60 min at 25°C in the presence or absence of the peptide mixture (automated solid-phase synthesis). The virus-peptide mixture, or virus alone, was incubated with CEM-SS cells for 60 min at 37°C to allow viral adsorption to the cells. Medium was removed and replaced with RPMI 1640 containing 10% fetal calf serum. Peptide was added during viral inoculation and maintained at the indicated concentration during growth to confluence. Cells were re-fed with fresh medium on day 3, and syncytia counted when cell confluency was reached 5 to 6 days later. Peptides did not affect cell viability, as assessed by rate of growth to confluence, cell number, or trypan blue exclusion. To confirm that the peptide CD4(76–94) mixture inhibited infection of CEM-SS cells as well as formation of syncytia scored as the primary assay readout (*11*), p24 was measured in cell supernatants on day 6. Duplicate cultures inoculated with $HTLV\text{-}III_B$ in the absence of peptide contained an average p24 concentration of 68 ng/ml. Duplicate cultures inoculated with $HTLV\text{-}III_B$ in the presence of 500 μ*M* CD4(76–94) peptide mixture ($V_n/V_0 = 0.003$) contained an average p24 concentration of 4.6 ng/ml. All peptides were tested at 5 to 500 μ*M* (0.5 to 500 μ*M* for ASTTTNYT). Several of the peptides were also tested for inhibition of $HIV\text{-}2_{NIH\text{-}Z}$ infection in this assay. Significant inhibition of $HIV\text{-}2_{NIH\text{-}Z}$ infectivity ($V_n/V_0 < 0.5$) was observed with the CD4(76–94) post-resin peptide mixture at a nominal concentration of 125 μ*M*.

The biologically active peptide mixture derived from synthesis of CD4(76–94) was fractionated by high-performance liquid chromatography (HPLC). The antisyncytial activity eluted separately from the desired peptide, as a distinct fraction that displayed a complex mass spectrum, suggesting a mixture containing derivatives of the parent peptide (Fig. 1). Mass spectrometric analysis of the major peak confirmed that it contained the desired peptide LKIEDSDTYICEVEDQKEE. This peak was without biological activity. To determine if the biological activity might result from derivatization of the parent peptide, purified inactive CD4(76–94) was derivatized by post-synthesis solution chemistry. The majority of the amino acid–protecting groups used for the synthesis of CD4(76–94) are benzyl esters or ethers (*9*). Thus benzylated peptides could result from incomplete deprotection of the parent peptide following synthesis. Accordingly, purified, inactive CD4(76–94) was chemically derivatized by benzyl or methylbenzyl addition to the peptide by using two different synthetic routes, each of which resulted in material with activity comparable to the original unpurified CD4(76–94) peptide mixture (*12*).

The cysteine residue within the sequence of CD4(76–94) is likely to be benzylated in the reaction performed (*12*). The importance of the cysteine residue was confirmed by the synthesis of serinyl and alanyl congeners of CD4(83–94) and CD4(76–94), and the phenylalanyl congener of CD4(76–94)amide. None of these peptide mixtures possessed detectable antisyncytial activity (Table 2). In view of the comparable activity of the CD4(76–94) synthetic mixture and the peptides benzylated by solution derivatization (*12*), we tested several *S*-benzyl cysteine congeners of CD4(76–94), and a series of COOH- and NH_2-terminal deletions

from this sequence, for their ability to inhibit HIV-induced cell fusion. The core sequence for antisyncytial activity was CD4(83–89) (Table 2). Biological activity requires the correct sequence of the core peptide, as well as derivatization, since a 19-residue peptide with the same composition but slightly altered sequence compared to CD4(76–94) had no antisyncytial activity (Table 1). The activity was attributable to peptide material, since proteolytic digestion completely abolished the antisyncytial activity of the active peptide preparations (see legend to Fig. 2).

Upon HPLC fractionation, the antisyncytial activity of the 12-residue *S*-benzyl CD4(83–94) peptide mixture eluted in a more hydrophobic region of the chromatogram than the parent compound (Fig. 2). Material in this region of the chromatogram, designated peak 7, gave a mass spectrometric profile consistent with a multiply derivatized peptide. Peak 7 was also tested as an inhibitor of cell fusion induced by several isolates of HIV-1, as well as SIV, and for its ability to inhibit HIV-induced cell fusion with the use of fresh human peripheral blood mononuclear cells (PBMC), rather than a $CD4^+$ lymphoid cell line, as the indicator cells in the fusion assay. In the first set of experiments, CD4(83–94)BZL (peak 7) was incubated in 96-well microtiter plates with 50,000 H9 cells infected with the viral isolates HIV-1_{TJ}, HIV-1_{DV}, HIV-1_{HXB2}, or $SIV_{UCDavis}$ for 30 min at 37°C. Levels of viral expression in each cell line were sufficient to allow formation of syncytia upon coculture with 50,000 $CD4^+$ indicator cells (VB cells for HIV-1–infected cellls; HUT-78 cells for SIV-infected cells) in a total volume of 100 μl of RPMI 1640 supplemented with heat-inactivated 10% fetal calf serum at a rate and frequency similar to those for the reference isolate HXB2 scored

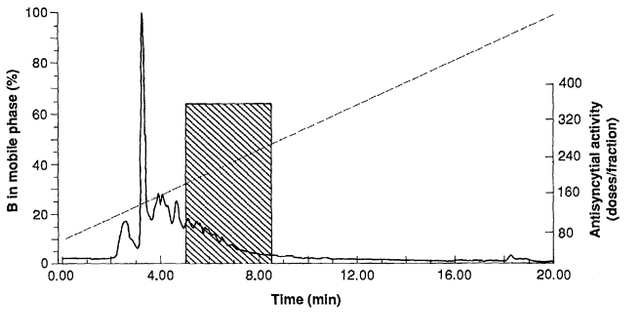

Fig. 1. Chromatographic fractionation of synthetic CD4(76–94). A representative chromatogram of 1.8 mg of CD4(76–94) on a Vydac C8 (10 × 250 mm) bonded-phase semipreparative column is shown. Material was post-resin CD4(76–94) dissolved in 10 m*M* ammonium acetate at *p*H 7.0. Mobile phase was (A) ammonium acetate buffer and (B) 20% ammonium acetate buffer/80% acetonitrile. The percentage of B in the mobile phase was varied as shown (dashed line). Material eluting at retention times 2 to 3, 3 to 4.5, 4.5 to 5, and 5 to 8.5 min was pooled from several semipreparative runs, lyophilized, weighed, and submitted to bioassay at about 500 to 32 μ*M* in the fusion assay described (*10*). Bioactivity (hatched bar) is expressed as doses of antisyncytial activity per fraction. One dose is the smallest amount of material necessary to completely inhibit fusion between 50,000 H9-HIV_{HXB2} cells and 50,000 VB indicator cells over an 18-hour period under standard assay conditions (*10*). Aliquots of the major peak (3 to 4.5 min retention time) and the area of the chromatogram in which bioactive material eluted (5 to 8.5 min retention time) were submitted to FAB-MS analysis as described (*21*). The major peak (3 to 4.5 min retention time) gave a parent fragment mass (M+H=2287) consistent with the mass of the desired peptide LKIEDSDTYICEVEDQKEE as well as a fragment of mass 2269, the mass of the parent fragment minus H_2O (18 atomic mass units). The biologically active material eluting at 5 to 8.5 min retention time exhibited a complex mass spectrum containing the parent M+H (2287) and multiple higher molecular weight peaks consistent with extensive derivatization of the parent peptide.

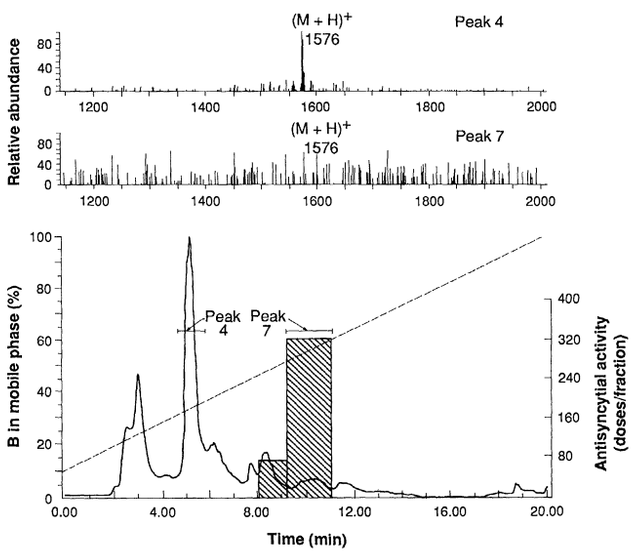

Fig. 2. Chromatographic fractionation of *S*-benzylCD4(83–94). The desired peptide was $TYIC_{bzl}$-EVEDQKEE where C_{bzl} indicates benzylation of Cys^{86} by insertion of the HF-stable t-Boc-*S*-benzyl cysteine in place of t-Boc-*S*-*p*-methylbenzyl cysteine in the solid-phase automated synthesis sequence. Ten milligrams of the post-resin peptide mixture was chromatographed as described in Fig. 1. Aliquots were taken for both FAB mass spectrometric analysis and bioassay (antisyncytial activity). Shown are the absorbance profile at 225 nm (broken line), the antisyncytial activity in each fraction (hatched boxes; dose defined as in Fig. 1), and the mass spectra of the major peak (peak 4) and the bioactive peak (peak 7) resolved by reversed-phase chromatography. The concentration of peak 7 material required to completely inhibit syncytium formation in the standard fusion assay (*10*) was ≤32 μ*M*. This concentration was calculated from the weighed mass of the material collected from this region of the chromatograph, and the molecular weight of the desired peptide. To demonstrate the peptide nature of the active component of CD4(83–94)BZL (peak 7), 100 μg of peak 7 material was dissolved in 50 μl of 0.1*M* ammonium bicarbonate to which was added 10 μg of Pronase in ammonium bicarbonate freshly prepared, or dissolved in buffer and inactivated by heating to 65°C for 2 min. Incubations were allowed to proceed at 37°C for 17 hours, after which time activity was quenched by incubation at 65°C for 2 min. Samples were lyophilized, and twice resuspended in water and re-lyophilized, before assay for antisyncytial activity as described (*10*). Potency of each preparation was determined in duplicate as described in Table 1. Peptide incubated with previously inactivated enzyme was active at 63 μ*M* (concentration to completely inhibit HIV-induced cell fusion). Peptide incubated without enzyme was active at 32 μ*M*. Peptide incubated with active enzyme which was then inactivated by heating at 65°C had no antisyncytial activity at the highest concentration tested (500 μ*M*).

Table 2. Antisyncytial activity of deletants and altered-sequence variants of CD4 Cys-benzyl(76–94) define a core antisyncytial peptide. Peptides were tested as post-resin peptide mixtures, as described (*9*). Subscript bzl indicates synthesis of the acid-stable benzyl thioether of cysteine at position 86. Antisyncytial activity was assessed as described in Fig. 1. Not active (NA) indicates no antisyncytial activity at early (6 hours) or late (24 hours) time points at the highest concentration tested, 500 μ*M*.

Sequence	Antisyncytial potency*
LKIEDSDTYIC_{bzl}EVEDQKEE	63 μ*M*
KIEDSDTYIC_{bzl}EVEDQKEE	125 μ*M*
EDSDTYIC_{bzl}EVEDQKEE	125 μ*M*
SDTYIC_{bzl}EVEDQKEE	125 μ*M*
TYIC_{bzl}EVEDQKEE	125 μ*M*
YIC_{bzl}EVEDQKEE	NA
LKIEDSDTYIC_{bzl}EVEDQKE	125 μ*M*
TYIC_{bzl}EVEDQKE	125 μ*M*
TYIC_{bzl}EVEDQK	250 μ*M*
TYIC_{bzl}EVEDQ	250 μ*M*
TYIC_{bzl}EVE	500 μ*M*
YIC_{bzl}EVE	NA
TYIAEVEDQKEE	NA
LKIEDSDTYIĀEVEDQKEE	NA
TYIS̄EVEDQKEE	NA
LKIEDSDTYIS̄EVEDQKEE	NA
LKIEDSDTYIF̄EVEDQKEE†	NA

*Antisyncytial potency is defined as the lowest concentration of peptide completely inhibiting fusion as described in Fig. 1. †Amide form.

as previously reported (*10*). CD4(83–94)BZL completely inhibited the formation of syncytia under these conditions at nominal concentrations of 63 μ*M* (HXB2, SIV) to 125 μ*M* (TJ, DV). In parallel experiments, human PBMC stimulated with phytohemagglutinin (1 μg/ml, Burroughs, Beckenham, England) for 48 hours were substituted for VB cells in the standard fusion assay. The post-resin peptide mixture $TYIC_{bzl}EVEDQKEE$ (see legend to Table 2) inhibited syncytium formation completely (18 hours of cocultivation with H9-HIV_{HXB2} cells) at approximately 63 μ*M* (Table 2). CD4(83–94)BZL was also tested for its ability to inhibit infection or fusion of CEM-SS cells (*11*) inoculated with cell culture supernatants from H9 cells infected with the HIV-1 strains HTLV-IIIB, RF-II, MN, and CC, which were selected for heterogeneity of the amino acid sequences of their envelope glycoproteins. When tested under the conditions described in Table 1, CD4(83–94)BZL (peak 7) completely inhibited infectivity and fusion of all four viral isolates at a nominal concentration of 125 μ*M*.

Mass spectrometric analysis confirmed that the major product of the synthesis of *S*-benzyl CD4(83–94) (designated peak 4) was the *S*-benzyl-cysteine peptide; this compound had no detectable biological activity (Fig. 2). These results suggested that antiviral activity either requires or tolerates derivatization of cysteine, and requires derivatization of at least one other site on the CD4(83–94) peptide $TYIC_{bzl}EVEDQKEE$. This hypothesis was confirmed by treatment of the biologically inactive peak 4 (Fig. 2), containing only authentic *S*-benzyl-cysteinyl CD4(83–94), with α-bromoxylene to effect further derivatization. This treatment resulted in material that blocked HIV-induced cell fusion (Table 3).

Since a limited number of amino acid residues could be derivatized in the parent 12-residue peptide CD4(83–94), a series of peptides was prepared by fluorenyl methyloxycarbonyl (FMOC) solid-phase synthesis using benzyl blocking groups on selected amino acid residues that are stable to FMOC cleavage conditions. Only the peptide in which Cys^{86} and Glu^{87} were benzylated yielded material with antisyncytial activity (Table 3). When purified to homogeneity by C8 followed by C18 reversed-phase HPLC the compound $TYIC_{bzl}E_{bzl}VEDQKEE$ was found to completely inhibit HIV-induced cell fusion at 125 μ*M*. The structure of purified $TYIC_{bzl}E_{bzl}VEDQKEE$ was confirmed by Edman degradation, fast-atom bombardment (FAB)–mass spectrometric analysis, and amino acid analysis. Since the peptide mixture contained within peak 7 (Fig. 2) was considerably more potent than $TYIC_{bzl}E_{bzl}VEDQKEE$, there must be other derivatives of CD4(83–94) that are more potent than, or synergistically enhance the potency of, this structurally defined peptide.

The antiviral activity of the CD4 peptide fragments described here is specific and is restricted to CD4-dependent interactions of cells with HIV proteins. CD4(76–94) at concentrations up to 250 μ*M* had no effect on HTLV-I–induced cell fusion in vitro (*13*), and complete inhibition of HIV-induced cell fusion was observed at a concentration of the CD4(76–94) peptide that did not affect the MLR response (Table 1). The mechanism of action of these CD4-derived peptides may therefore involve competitive blockade of viral attachment to CD4 via peptide binding to the CD4-combining region of the HIV gp120 glycoprotein. Consistent with this proposed mechanism of action, the partially purified CD4(83–94)BZL (peak 7) peptide blocked fusion between HIV-infected T cells and CD4-expressing T cell lines or CD4-expressing peripheral blood–derived cells, independent of the isolate of HIV used in the assay (see Table 2), blocked infection of $CD4^+$ lymphoid cells by both HIV-1 and HIV-2 (see Table 1), and also blocked the CD4-dependent fusion induced by SIV (see Table 2) (*14*).

In the native CD4 molecule, Cys^{86} is linked to Cys^{18} by a disulfide bond (*15*), which may impose conformational constraints on the peptide in the region of Cys^{86} that allow interaction with the HIV envelope glycoprotein (*16*). Derivatization of the peptide fragment CD4(83–94) may mimic this conformation, allowing the peptide to bind the viral envelope protein. Small CD4-derived peptides with antisyncytial activity should allow the design of antiviral agents with access to organ compartments that the larger CD4 molecule, even in soluble form (*17*), might not penetrate.

Table 3. Multiple benzylation of CD4(83–94) imparts antisyncytial activity to the peptide. The antisyncytial activity of five preparations of CD4(83–94), variously derivatized, were compared. The preparations were: (A) post-resin mixture after solid-phase synthesis of the desired peptide TYIC(*S*-benzyl)EVEDQKEE; (B) HPLC fractionation of the peptide mixture in A, peak 4 obtained as in Fig. 2; (C) liquid-phase derivatization of B, afforded by addition of 7.1 μmol of α-bromoxylene to 2.2 μmol of peak 4 dissolved in 1.5 ml of triethylamine followed by stirring at room temperature for 16 hours, removal of volatile material under vacuum, dissolution in 10 m*M* ammonium acetate, *p*H 7.0, extraction with one volume of chloroform, and repeated lyophilization of the resultant aqueous phase [after Erickson and Merrifield (*18*)]; (D) HPLC fractionation of the peptide mixture in A, peak 7 obtained as in Fig. 2; (E) the post-resin mixtures obtained by FMOC syntheses of several underivatized, monoderivatized, and diderivatized congeners of CD4(83–94) as described (*19*); (F) FMOC synthesized $TYIC_{bzl}E_{bzl}VEDQKEE$, purified by sequential HPLC fractionation on C8 and C18 columns, with structure and purity confirmed by analytical HPLC, amino acid sequencing, and definitive fragment ion series acquired in both positive and negative ion modes in FAB-MS analysis (*20*).

Material assayed	Nominal potency
(A) *S*-benzyl-CD4(83–94) peptide mixture	125 μ*M*
(B) Peak 4	NA
(C) Peak 4, solution-derivatized	250 μ*M*
(D) Peak 7	<32 μ*M*
(E) FMOC CD4(83–94) peptides	
TYICEVEDQKEE	NA
$TYIC_{bzl}EVEDQKEE$	NA
$TY_{Br\text{-}Z}IC_{bzl}EVEDQKEE$	NA
$TYIC_{bzl}EVE_{bzl}DQKEE$	NA
$TYICE_{bzl}VE_{bzl}DQKEE$	NA
$TYIC_{bzl}E_{bzl}VEDQKEE$	200 μ*M*
(F) Purified $TYIC_{bzl}E_{bzl}VEDQKEE$	125 μ*M*

REFERENCES AND NOTES

1. E. M. Ross and A. G. Gilman, in *The Pharmacological Basis of Therapeutics*, A. G. Gilman, L. S. Goodman, T. W. Rall, F. Murad, Eds. (Macmillan, New York, 1985), pp. 35–48; E. J. Ariens and A. J. Beld, *Biochem. Pharmacol.* **26**, 913 (1977).
2. E. L. Reinherz, P. C. Kung, G. Goldstein, S. F. Schlossman, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4061 (1979); E. L. Reinherz and S. L. Schlossman, *Cell* **19**, 821 (1980); J. A. Ledbetter *et al.*, *J. Exp. Med.* **153**, 310 (1981).
3. E. G. Engleman, C. J . Benike, E. Glickman, R. L. Evans, *J. Exp. Med.* **153**, 193 (1981); E. G. Engleman, C. J. Benike, C. Metzler, P. A. Gatenby, R. L. Evans, *J. Immunol.* **130**, 2623 (1983); S. C. Meuer, S. F. Schlossman, E. L. Reinherz, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4395 (1982); W. E. Biddison, P. E. Rao, M. A. Talle, G. Goldstein, S. Shaw, *J. Immunol.* **131**, 152 (1983).
4. A. M. Krensky, C. Clayberger, C. S. Reiss, J. L. Strominger, S. J. Burakoff, *J. Immunol.* **129**, 2001 (1982).
5. A. G. Dalgleish *et al.*, *Nature* **312**, 763 (1984); D. Klatzman *et al.*, *ibid.*, p. 767; P. J. Maddon *et al.*, *Cell* **47**, 333 (1986); M. O. McClure *et al.*, *Nature* **330**, 487 (1987); M. Kannagi, J. M. Yetz, N. L. Letvin, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7053 (1985).
6. J. S. McDougal *et al.*, *Science* **231**, 382 (1986).
7. J. D. Lifson, G. R. Reyes, M. S. McGrath, B. S. Stein, E. G. Engleman, *Science* **232**, 1123 (1986); J. D. Lifson *et al.*, *Nature* **323**, 725 (1986); J. Sodrowski, W. C. Goh, C. Rosen, K. Campbell, W. Haseltine, *ibid.*, p. 470.
8. P. J. Maddon *et al. Cell* **42**, 93 (1985).
9. Synthetic peptides and peptide derivatives corresponding to regions of the deduced amino acid sequence of human CD4 have been identified by the amino acid residue numbers of CD4 (*8*). Synthesis of peptides and their side products and derivatives was carried out on an Applied Biosystems model 430A peptide synthesizer, according to the principles of Merrifield [G. Barany and R. B. Merrifield, in *The Peptides: Analysis, Synthesis, Biology*, E. Grass and J. Meinhofer, Eds. (Academic Press, New York, 1979), vol. 5, pp. 1–284]. Side chain protection and cleavage of peptide products from the resin was

carried out by standard procedures to be described elsewhere and available from the corresponding author on request. All peptides were tested as post-resin material, which represents a peptide mixture containing 65 to 95% of the desired peptide, as well as partially deprotected side products.

10. The HIV-induced cell fusion assay was carried out as described by J. D. Lifson *et al.* (7) and J. D. Lifson *et al.*, *J. Exp. Med.* **164**, 2102 (1986). Nominal peptide concentrations, at which complete blockade of HIV-induced cell fusion was observed after 18 hours of incubation of HIV- or SIV-infected H9 cells and CD4-expressing indicator cells, are given in this report.
11. P. L. Nara, W. C. Hatch, N. M. Dunlop *et al.*, *AIDS Res. Hum. Retrovir.* **3**, 283 (1987).
12. Five milligrams of CD4(76–94) purified by HPLC as described in Fig. 1 was submitted to chemical derivatization as described in Table 3, (C). The resultant peptide derivatives were evaporated to dryness, reconstituted in water, extracted with one volume of chloroform, and the aqueous phase lyophilized and tested for antisyncytial activity in the standard assay (*10*). Potency is expressed as the lowest concentration of the peptide mixture (nominal concentration based on mass of the input peptide and formula weight of the parent peptide LKIEDSDTYICEVEDQKEE) capable of complete inhibition of HIV_{HXB2}-induced cell fusion (*10*). Under these conditions, CD4(76–94), inactive by itself, was, upon treatment with α-bromoxylene or α-iodotoluene, active to inhibit HIV-induced cell fusion at 125 and 60 μ*M*, respectively.
13. The effects of peptides on HTLV-I–induced cell fusion were tested by means of a cocultivation assay with the X/C cell line being used as an indicator and the cell line C91/PL as the source of infected cells. We incubated 2.5×10^4 C91/PL cells for 30 min in 50 μl of medium (RPMI 1640 with 10% heat-inactivated fetal calf serum), along with 100 μl of medium, or sufficient peptide [CD4(76–94) post-resin peptide mixture] dissolved in 100 μl of medium to yield a final nominal concentration of 250 μ*M*. An equal number of indicator cells was added, and after 18 hours of cocultivation, cells were fixed with ethanol, stained with Giemsa stain, and HTLV-1–induced syncytia (more than four nuclei within a common cell membrane) were enumerated under an inverted microscope. Mean values (± standard error) for four replicate determinations for each condition were: no peptide added, 135 ± 6 syncytia per well; CD4(76–94) post-resin peptide mixture, at 250 μ*M*, 128 ± 4 syncytia per well.
14. H. Kornfeld, N. Riedel, G. A. Viglianti, V. Hirsch, J. I. Mullins, *Nature* **326**, 610, 1987; L. J. Lowenstine *et al.*, *Int. J. Cancer* **38**, 563 (1986).
15. B. J. Classon *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4499 (1986).
16. Q. L. Sattentau, A. G. Dalgleish, R. A. Weiss, P. C. L. Beverley, *Science* **234**, 1120 (1986); M. Kolwalski *et al.*, *ibid.* **237**, 1351 (1987); L. Lasky *et al.*, *Cell* **50**, 975 (1987).
17. D. H. Smith *et al.*, *Science* **238**, 1704 (1987); R. A. Fisher *et al.*, *Nature* **331**, 76 (1988); R. E. Hussey *et al.*, *ibid.*, p. 78; K. C. Deen *et al.*, *ibid.*, p. 82; A. Traunecker, W. Luke, K. Karjalainen, *ibid.*, p. 84; E. A. Berger, T. R. Fuerst, B. Moss, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2357 (1988).
18. B. W. Erickson and R. B. Merrifield, *J. Am. Chem. Soc.* **95**, 11 (1973).
19. This series of peptides was synthesized on an ABI model 430A peptide synthesizer, using FMOC amino acid HOBT esters except for those residues in which a protecting group was desired. Amino acids in these cases were FMOC–cysteine benzyl ether, FMOC–threonine bromocarbobenzoxyl ester, FMOC–glutamate benzyl ether, and FMOC–tyrosine benzyl ether. Peptides were synthesized on a *p*-hydroxymethylphenoxyacetic acid resin and cleaved with 95% trifluorocetic acid.
20. T. D. Lee, in *Methods of Protein Microcharacterization*, J. E. Shively, Ed. (Humana, Clifton, NJ, 1986), pp. 403–440.
21. A. M. Buko and B. A. Fraser, *Biomed. Mass Spectrum* **12**, 577 (1985).

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